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Example 3
Recombinant Modified Vaccinia Ankara (MVA)
Expressing the TAA lac Z and IC-2

Modified Vaccinia Ankara (MVA) strain is used as the expression vector. MVA was derived from vaccinia virus (WT) by over 570 serial passages in chicken embryo fibroblast cells (CEF) (Mayr, A. et al. Infection 3:6-14, 1975). The resulting MVA strain lost the capacity to productively infect mammalian cells (Altenburger, W. et al. Arch. Virol 105:15-27, 1989; Meyer, H. et al. J. Gen. Virol 72:1031-1038, 1991). The expression of late, as well as early, viral genes is unimpaired in human cells despite the inability of MVA to produce infectious progeny (Sutter and Moss, Proc. Natl. Acad. Sci. USA 89:10847-10851, 1992).

An insertion plasmid is constructed with the lac Z gene and the B7.1 gene under the control of the vaccinia virus late promotor P11 to allow homologous recombination at the site of a naturally occurring 3500-base-pair deletion within the MVA genome. MVA recombinants are isolated and propagated in permissive avian cells as described in Sutter and Moss 1992 and Sutter et al. Virology 1994. The expression of β-gal and B7.1 upon infection of nonpermissive human cells is detected by methods described herein.

Example 4
Effect Of Inoculation Of Various
Recombinant Vaccinia Virus Constructs
On Primary And Secondary Responses
Against Vaccinia Or β-Galactosidase

Materials and Methods

5 BALB/c $(H-2^d)$ mice per group were inoculated i.v. or s.c. with different doses the following vaccinia recombinant (rVV): IL-2 rVV; GM-CSF-rVV; TNFα-rVV; IFNγ-rVV.

In all these constructs the mouse model tumor

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associated antigen, β-Gal was under the control of the
p7.5-kD (E/L) promoter (Cahran, M.A. et al _J. Virol._ 54
(No. 1):30-37, 1995) while the cytokine production was
driven by $p_{synthetic\ late}$ promoter (Davidson et al _Nucleic Acid
Research_ 18 (No. 14):4285-5286, 1991). All these rVV were
generated by recombination in the TK region of vaccinia
using the plasmid pMJ601 described in Davison et al
Nucleic Acids Research 18 (No. 14):4285-5286, 1991.
VJS6

In this construct, containing the E6 protein from
Human Papilloma Virus (HPV) a pE/L promoter (the
"synthetic superpromoter") is placed just upstream the
p7.5 promoter controlling the β-Gal expression and
oriented in the same direction.
Control Vaccine

Control vaccinia (crude 19, NP-VV) does not
expressing β-Galactosidase.
Primary in vivo Responses and Secondary in vitro Responses

Female BALB/c mice, 8-12 weeks old, were obtained
from the Animal Production Colonies, Frederick Cancer
Research Facility, NIH, Frederick, MD. Primary lymphocyte
populations were generated by injecting BALB/c mice i.v.
or subcutaneously with 10^6-10^7 plaque forming units (pfu)
of recombinant virus (2 doses in some cases). To assay
for primary _in vivo_ responses spleens were harvested on
day six, dispersed into a single cell suspension and
tested for their ability to lyse β-gal expressing and
control targets in a six hour ^{51}Cr release assay.
Secondary _in vitro_ effector populations were generated by
harvesting the spleens of mice 21 days after immunization
with recombinant virus and culturing single cell
suspensions of splencytes in T-75 flasks (Nunc, Denmark)
at a density of 5.0 x 10^6 splenocytes/ml. with 1 μg/ml of
antigenic peptide in a total volume of 30 ml of culture
medium consisting of RPMI 1640 with 10% fetal calf serum
(both from Biofluids) that contained 0.1 mM non-essential

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amino acids, 1.0mM sodium pyruvate (both from Biofluids)
and 5 x 10⁻⁵ M 2-mercaptoethanol (GIBCO/BRL, Rockville, MD)
in the absence of IL-2. Six days later splenocytes were
harvested and washed in culture medium before testing in a
^{51}Cr release assay.

^{51}Cr release assay

Six-hour ^{51}Cr release assays were performed as
previously described (Restifo, N. et al. J. Exp. Med.
177:265, 1993). Briefly, 1 x 10⁶ target cells were
incubated with 200mCi Na^{51}CrO₄(^{51}Cr) for ninety minutes.
Peptide pulsed targets were incubated with 1 μg/ml (which
is roughly 1μM) of antigenic peptide (for β-gal =
TPHPARIGL peptide) during labeling as previously described
(Restifo, N. et al. J. Immunol 147:1453, 1991). Target
cells were then mixed with effector cells for six hours at
the effector to target (E:T) ratios indicated. The amount
of ^{51}Cr released was determined by γ-counting and the
present specific lysis was calculated from triplicate
samples as follows: [(experimental cpm - spontaneous
cpm)/(maximal cpm - spontaneous cpm)] x 100.

In vivo protection and treatment studies

For *in vivo* protection studies, mice were immunized
with recombinant virus 21 days before a subcutaneous
challenge with 10⁴ tumor cells or an intravenous challenge
with 5 x 10⁵ tumor cells, as previously described (31).
After tumor challenge all mice were randomized. Mice
receiving subcutaneous tumor were measured twice a week.
When tumors developed, they all grew progressively and
were lethal. Mice were euthanized, however, when they
were moribund. All mice that appear as long term
survivors had no palpable tumor. Mice receiving
intravenously administered tumor were euthanized on day 12
and randomized before counting lung metastases in a
blinded fashion as previously described (2).

For *in vivo* treatment studies, un-irradiated BALB/c

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mice were challenged with either 10^5 or 5×10^5 tumor cells
intravenously in order to establish pulmonary metastases.
Mice were subsequently vaccinated with 10^7 PFU of the
designated recombinant virus intravenously or
subcutaneously on days three or six. Mice receiving
intravenously administered tumor were euthanized on day 12
and randomized before counting lung metastases in a
blinded fashion.

The following targets were used in each assay:

CT26 = murine H-2^d adenocarcinoma

CT26 gal = murine H-2^d adenocarcinoma expressing β-
Gal. CT26 gal also expresses higher levels of class I MHC
molecules.

E22 = murine H-2^d thymoma expressing β-Gal.

CT26 + peptide = CT26 pulsed with TPHPARIGL (876-884)
L^d-restricted β-gal peptide

CT26-vac = CT26 infected with crude 19 vaccinia

Lytic Units 30% were calculated for the anti-vaccinia
cytotoxic response. Lytic units 30% (L.U. 30) indicate
the number of effector cells necessary to give a 30% lysis
of 10,000 target cells.

Example 5
Cytokine Secretion After Infection
Of BSC-1 Cells With Various
Recombinant Vaccinia Virus Constructs

Duplicate wells of 10^5 BSC1 in 1 ml of 2.5% fetal
calf serum (FCS) medium RPMI 1640 with antibiotics (24
well plates) were infected with VJS6, rVV-IL-2, rVV-GM-
CSF, rVV-IFNγ or rVV-TNFα. At the end of incubation at
27°C for 24 hours, supernatants were removed, centrifuged,
and the concentration of cytokine determined as shown in
Table 1. Values are expressed in pg/ml.

The concentration of each cytokine was determined
using commercially available detection kits for GM-CSF,
IFNγ; IL-2 and TNFα.

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The results show that high concentrations of each cytokine were detected. The highest concentration of cytokine produced was IL-2 from BSC-1 cells infected with rVV-IL2 (Table 1).

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Table 1

DETECTION OF CYTOKINES SECRETED AFTER
INFECTION OF BSC1 CELLS WITH DIFFERENT rVV

rVV	MOI 12h				MOI 36h			
	1:1	0.1:1	0.01:1	TITER*	1:1	0.1:1	0.01:1	TITER*
GM-CSF detection kit								
VJS6	<15.6	<15.6	<15.6	-	<15.6	<15.6	<15.6	-
IL-2	<15.6	<15.6	22	-	<15.6	<15.6	<15.6	-
GMCSF	>250	>250	>250	5.1×10^5	>250	>250	>250	4.2×10^6
IFNγ	<15.6	<15.6	<15.6	-	<15.6	<15.6	<15.6	-
TNFα	<15.6	<15.6	<15.6		<15.6	<15.6	<15.6	
IFNγ detection kit								
VJS6	<47	<47	<47	-	<47	<47	<47	-
IL-2	<47	<47	<47	-	<47	<47	<47	-
GMCSF	<47	<47	<47	-	<47	<47	<47	-
IFNγ	>12000	9000	1000	56000	>12000	>12000	>12000	>67000
TNFα	<47	<47	<47	-	<47	<47	<47	-
IL-2 detection kit								
VJS6	<34	<34	<34	-	<34	<34	<34	-
IL-2	>850	>850	>850	2×10^6	>850	>850	>850	3.9×10^6
GMCSF	<34	<34	50	-	54	63	110	-
IFNγ	<34	121	135	-	94	97	132	-
TNFα	<34	<34	<34	-	<34	<34	<34	-
TNFα detection kit								
VJS6	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
IL-2	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
GMCSF	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
IFNγ	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
TNFα	2050	310	295	2050	>2450	>2450	>2450	135000

a.   Supernatants from 1:1 MOI (multiplicity of infectivity) infected cells, that were expected to
     exceed the highest point of the standard curve, were serially diluted (1:10 dilution) to
     calculate the precise cytokine concentration.

b.   Only one of the two wells was positive.

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Example 6

β-Galactosidase Production After BSC1 Cell
Infection With Various Vaccinia Virus Constructs

One X10^5 BSC1 cells were infected with 1:1 MOI of
either rVV-VJS6, rVV-IL2, rVV-GM-CSF- rVV-TNFα or rVV-
IFNγ. At this MOI the cytopathic effect was less
pronounced at time end of incubation, and presumably no
leakage of the enzyme had occurred. Pelleted cells were
subject to three freeze-thaw rounds to release the
cytoplasm content, cellular debris was removed and the
supernatant used for detection of galactosidase activity
on the substrate O-nitrophenyl-β-D-galactopyranoside
(ONPG) using a Promega Kit No. E2000 (Promega, Madison,
Wisconsin). One unit of galactosidase hydrolyze 1μM ONPG
per minute at pH 7.5 at 37°C. Duplicate wells were run on
each sample. The results from two different experiments
are shown in Figure 1. The control vaccinia, NP-VV, did
not express β-gal. VJS6 samples were serially diluted to
fit within the depicted range. The results show a 1 log
difference in the β-Gal enzymatic activity of VJS6
compared to the other recombinant vaccinia virus. The
results as shown in Figure 1 parallel with the kinetics of
blue staining with X-gal in plaque assays (data not
shown).

Example 7

Primary Response Of Mice At Day 6 After
Injection I.V. With Various Vaccinia Virus Constructs

5 BALB/c mice were injected intravenously (I.V.) with
5x10^6 plaque forming units (pfu) of the following
recombinant vaccinia virus: VJS6, IL2-rVV, GM-CSF-rVV,
TNFα-rVV OR IFNγ-rVV. At day 6, the spleens were removed
and tested against the following target cells as described
in Example 4: CT26, CT26 peptide, CT26 gal, E22, and CT26
vac.

Figure 2 shows the peak primary CTL response. IL-2
and GM-CSF produced by rVV during infection notably

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enhanced the primary CTL response against vaccinia
determinants. The spleen cells also gave a weak primary
response toward β-gal.

Example 8
Secondary Cultures From Mice
Infected I.V. With Various
Recombinant Vaccinia Virus Constructs

Mice were infected with various recombinant vaccinia
virus constructs as described in Example 4. Splenocytes
were harvested at day 6 or day 14 after inoculation with
the rVV. Secondary cultures were generated by 6 days
incubation of 6×10^5 splenocytes/ml in complete medium
containing 1 μg/ml TPHPARIGC peptide as indicated in
Example 4. The results showed that CTL from VJS6, IL2-rVV
and GM-CSF-rVV vaccinated mice could not be restimulated
with an β-gal peptide 6 days after viral injection (Figure
3). The TNF-rVV response of CTLs was characterized by the
induction of a long-lasting non-specific cytotoxicity
(Figures 3 and 4). The GM-CSF production had a negative
effect on the anti-β-gal immune response of CTLs.

Example 9
Primary Response Of Mice At Day 6 After Injection
S.C. With Various Recombinant Vaccinia Virus Constructs

5 BALB/c mice were injected subcutaneously (S.C.)
with 5×10^6 pfu of the following recombinant vaccinia
virus: VJS6, IL2-rVV, GM-CSF-rVV, TNFα-rVV or IFNγ-rVV.
At day 6, the spleens were removed and tested against the
following target cell as described in Example 4: CT26,
CT26 peptide, CT26 gal, E22 and CT26 vac.

Figure 5 shows in all cases, each cytokine helped to
increase the response to vaccinia in comparison to VJS6
using the CT26 target, with the IL-2-rVV treatment being
the highest. No lysis was obtained with the other target
cells. This indicates that the kinetics of the immune
response induced by S.C. inoculation may be different or
delayed in comparison to I.V. inoculation. However,

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higher doses and/or different time intervals is expected
to increase the magnitude of the primary response against
vaccinia using the subcutaneous route of inoculation.

The lytic units (L.U.) 30% was determined by the
number of effector cells necessary to give 30% lysis of
10,000 target cells using the CT26-vac cells as targets,
Figure 6 shows the results of this titration. The
L.U./spleen (x10^3) of each rVV is as follows: Gm-CSF=
124.3; VJS6 - 14.9; IFN-γ - 5.06; TNFα = 2.88 and IL-2 =
96.2.

Example 10
Secondary Cultures From Mice
Infected S.C. With Various
Recombinant Vaccinia Virus Constructs

Mice were infected with various recombinant vaccinia
virus constructs as described in Example 4. Splenocytes
were harvested at day 14 after inoculation with rVV.
Secondary cultures were generated by 6 days incubation of
6x10^5 splenocytes/ml in complete medium containing 1 μg/ml
TPHPARIGC peptide as indicated in Example 4. The results
as depicted in Figure 7 show a high level of non-specific
response to each target cell regardless of the effector
cell.

Example 11
Treatment Of Established Pulmonary
Metastases With Recombinant Vaccinia
Viruses Secreting Different Cytokines

The three-day pulmonary metastases mouse model was
used to evaluate the efficacy of treatment using rVV.
Nontreated mice normally succumb to the metastases in 11-
14 days using this model system.

Mice were injected I.V. on day 0 with 5x10^5 tumor
cells (CT26 or CT26 β gal). Three days later they
received an I.V. injection of 5x10^5 PFU of VJS6, IL-2-rVV,
GM-CSF-rVV, IFNα-rVV, TNFγ-rVV or Hanks' Balanced Salt
Solution (HBSS) (control mice). The mice were randomized,
and the lungs were harvested after 12 days. The number of

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metastases was determined by counting in a blinded
fashion.

Table 2 shows that moderate doses of IL2 produced by
the rVV was effective in treatment of 3 day old metastases
induced by CT26 β gal.

Table 2

DAY 3 TREATMENT OF ESTABLISHED PULMONARY METASTASES WITH
RECOMBINANT VACCINIA VIRUSES SECRETING DIFFERENT CYTOKINES

rVV treatment	CT26		CT26 βgal	
	average # metastases	metastases/ mouse	average # metastases	metastases/ mouse
none*	>500	>500 x 5	>500	>500 x 5
VJS6	>500	>500 x 5	186	162, 116, 115, 362, 175
IL-2 rVV	452.4	500 x 4, 262	11.4	13, 2, 24, 12, 7
GM-CSF rVV	470.2	500 x 4, 351	373.6	500 x 2, 405, 283, 179
IFNγ rVV	>500	>500 x 5	232.8	280, 97, 190, 260, 337
TNFα rVV	>500	>500 x 5	361.2	>500 x 2, 389, 258, 159

* control mice were injected with HBSS alone

Example 12

Treatment Of Established Pulmonary Metastases
With Recombinant Vaccinia Virus Secreting
Different Cytokines Plus Exogenous IL2

Mice were injected with tumor cells and rVV as
indicated in Example 4. Treatment with exogenous rIL-2
(15,000 cetus units, twice a day, I.P.) was started 6 h
after rVV injection and protracted for 5 days. Lungs were
harvested after 12 days.

Moderate doses of IL2 (produced by rVV or exogenously
administered together with rVV) were effective in
treatment of 3 day old metastases induced by CT26 β- gal.
(Table 3)

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Table 3

DAY 3 TREATMENT OF ESTABLISHED PULMONARY METASTASES WITH
RECOMBINANT VACCINIA VIRUSES PRODUCING DIFFERENT CYTOKINES PLUS
EXOGENOUS IL-2

rVV treatment	CT26		CT26 βgal	
	average # metastases	metastases/ mouse	average # metastases	metastases/ mouse
none[a]	>500	>500 x 5	>500[b]	>500 x 5
VJS6	>500	>500 x 5	405.2	>500 x 3, 267, 259
IL-2 rVV	437.4	500 x 3, 267, 420	127.4[c]	184, 7, 126, 91, 229
rIL-2	>500	>500 x 5	>500	>500 x 5
rIL-2 + VJS6	406.4	>500 x 3, 298, 234	20.8[c]	43, 6, 0, 52, 3
GM-CSF rVV	413.2	>500 x 3, 163, 403	500	500 x 5
IFNγ rVV	447.6	>500 x 4, 238	412.2	500 x 4, 66

[a] control mice were injected with HBSS alone
[b] all mice in this group died between days 11 and 12 (before lung harvest)
[c] p value between the data in the frame is 0.07

Example 13

Active Immunotherapy Using
Exogenous IL-2 Together With rVV
Expressing A Tumor Associated Antigen

A recombinant vaccinia virus expressing a Tumor
Associated Antigen (TAA) was constructed as described in
Example 1.

Mice were injected with 5 x 10^{5} of CT-26 expressing
β-gal via an I.V. route of administration. Three days
later the mice were injected with rVV-TAA-β-gal, alone or
in combination with exogenous IL2. [High dose (HD)
exogenous IL-2 (100,000 I.U., I.P., BID x 3d) or low dose
(IL) IL-2 (15,000 I.U., I.P., BID x 3d)] was administered
as indicated and then randomized.

The number of mice surviving and survival time in
days was monitored. Those mice receiving rVV-TAA plus
exogenous IL2 survived. (Figure 8)

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Example 14

Figure 9 shows the survival of mice with non-β-gal
expressing tumor established after immunotherapy with
different recombinant vaccinia vectors. Mice were
injected with 5 x 10^5 CT 26 tumor cells (non-β-gal) via
I.V. route of administration. On day three they were
injected with 10^3 pfu of the following rVV which also
contained the β-gal gene: IL-2rVV, TNFα-rVV, VJS6, GM-
CSFrVV. The number of mice surviving and survival time in
days was monitored. No difference in survival was
observed in any of the treatment groups.

Figure 10 shows survival in mice with an established
β-gal expressing tumor, CT26.25 after immunotherapy with
different recombinant vaccinia vectors. Mice were
injected with 5 x 10^5 CT26.25 (β-gal expressing) via I.V.
route of administration. On day three they were injected
with 10^3 pfu of the rVV described above. The results show
that a clear survival advantage was conferred on those
mice treated with the IL-2rVV (also containing the β-gal
gene) compared to treatment with any other rVV.

Example 15
Active Treatment Of Established
Pulmonary Metastases Using
B7 - rVV Construct

All of the following recombinant Vaccinia vectors
were constructed by placing both the cytokine gene and the
TAA gene in the TK region of the vaccinia vector genome.
Promotors varied based on the plasmid used for
recombination. The majority of the constructs used the
P7.5K promotor.

The three-day pulmonary metastases model was used as
described in Example 4. Five x 10^5 tumor cells were
injected into mice. Three days later 10^6-10^7 pfu of each
vector was injected I.V. Lungs were harvested on day 12.

The results as depicted in Figure 11 and 12 showed
that the rVV vectors encoding ß-gal and the

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immunostimulatory molecule, B7.1, IL-2 or ICAM-1
profoundly inhibited pulmonary metastases in an antigen
specific manner.

The primary response of the mice on Day 9 after
injection with tumor cells was determined. The results
are depicted in Table 4. The percent lysis of ^{51}Cr labeled
target tumor cells is recorded. A significant primary
immune response was seen with multiple recombinant
vaccinia vectors, most notably B7.1rVV, which correlates
with the _in vivo_ treatment response.

Table 4
Primary Response of Mice 6 Days After Injection
I.V. With Various Vaccinia Virus Constructs

Tumor cells					
rVV (pfu) Construct	Effector Target	CT26	CT26/P	C25	CT26/V69
B7.1	100:1	-3.86*	82.99	81.45	21.69
	33:1	-4.86	91.86	69.01	14.49
	11:1	-6.10	61.64	71.75	7.33
	37:1	-8.61	53.99	54.22	2.19
	1.2:1	-9.53	39.93	40.87	3.25
	04:1	-16.57	27.37	26.74	-0.15
B7.1 10^6	100:1	2.75	68.18	5270	70.75
	33:1	-2.05	84.93	66.76	82.89
	11:1	-2.85	41.27	73.12	56.05
	37:1	-3.76	41.93	350.06	42.19
	12:1	-8.05	26.24	31.70	44.97
	0.4:1	-8.52	20.50	35.49	37.13
NA 10^7**	100:1	4.04	79.02	69.65	14.06
	33:1	1.70	72.66	82.36	10.65
	37:1	-1.01	51.75	63.41	7.53
	11:1	-2.75	35.65	42.33	0.37
	12:1	-4.88	34.98	28.49	-11.67
	0.4:1	-6.57	6.14	20.02	-13.66
NA 10^6	100:1	27.40	88.40	121.19	31.48
	33:1	13.47	74.16	112.42	1.41
	11:1	0.53	41.16	97.33	-4.07
	3.7:1	-0.35	8.95	86.27	1.58
	1.2:1	-0.43	-2.05	69.52	371
	04:1	-4.06	-2.59	40.13	-4.37

MVA 10^8	100:1	12.76	27.43	65.08	12.10
	33:1	8.52	17.62	61.83	8.37
	11:1	3.45	2.95	49.38	4.34
	37:1	0.06	5.76	28.12	3.13
	1.2:1	-1.30	2.43	28.05	-3.60
	0.4:1	-4.26	-0.61	16.78	-7.65
MVA 10^7	100:1	17.54	71.07	46.31	0.14
	33:1	13.81	70.97	37.10	1.10
	11:1	6.36	44.59	35.00	-0.30
	3.7:1	-2.29	17.29	28.58	-6.94
	1.2:1	-3.54	2.82	27.91	-0.76
	0.4:1	-17.53	-6.63	19.66	-9.87
Kb 10^7	100:1	24.83	20.58	33.37	34.47
	33:1	20.74	15.35	19.42	34.75
	11:1	12.61	-10.25	9.73	27.77
	37:1	9.82	-11.37	8.51	17.51
	1.2:1	-5.98	-18.23	8.58	17.01
	0.4:1	-10.88	-29.04	5.55	9.10
Kb 10^6	100:1	11.60	28.14	42.96	-0.32
	33:1	-2.37	16.79	21.60	-1.60
	11:1	-7.83	16.12	-1.67	-7.08
	37:1	-12.70	8.51	-3.28	-11.12
	1.2:1	-18.17	6.15	-11.68	-9.36
	0.4:1	-24.72	-4.83	-17.02	-14.28
Ld 10^7	100:1	-9.69	86.06	92.89	-5.1
	33:1	-32.65	33.05	53.22	-8.54
	11:1	-39.51	35.26	46.63	-19.29
	37:1	-42.44	15.50	24.28	-17.91
	1.2:1	-47.50	2.17	12.71	-17.45
	0.4:1	-51.77	-1.65	9.44	-30.42
Ld 10^6	100:1	28.10	56.29	30.10	16.62
	33:1	16.09	41.69	26.49	-5.75
	11:1	-1.22	24.49	24.50	-17.06
	37:1	-3.46	24.04	18.81	-21.05
	1.2:1	-9.65	8.65	13.16	-20.82
	0.4:1	-17.10	2.42	8.34	-27.53
VJS6 10^7	100:1	29.38	33.59	73.98	22.03
	33:1	24.69	31.48	72.14	24.43
	11:1	20.17	27.87	61.82	13.66
	37:1	16.83	23.47	57.46	7.24

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	1.2:1	8.44	17.93	51.60	4.11
	0.4:1	4.01	19.12	33.13	-5.46
VJS6 10^6	100:1	1.53	63.88	63.8	45.57
	33:1	-23.69	35.15	36.13	39.89
	11:1	-27.32	29.65	27.61	29.29
	37:1	-41.54	21.54	21.57	24.26
	1.2:1	-45.02	18.21	16.21	21.06
	0.4:1	-52.85	14.18	8.78	18.50
WB_2m 10^7	100:1	3.50	11.12	23.52	4.69
	33:1	0.32	11.95	10.52	3.46
	11:1	-10.09	5.10	7.51	-2.53
	37:1	-21.06	0.22	1.10	-2.51
	1.2:1	-28.28	-3.99	-3.04	-4.26
	0.4:1	-30.42	-7.84	-11.03	-7.36
WB_2m 10^6	100:1	25.09	16.18	22.86	1.54
	33:1	25.61	6.04	19.48	-2.55
	11:1	22.77	-1.80	14.43	-11.26
	37:1	9.18	-8.25	11.40	-19.36
	1.2:1	3.90	-19.60	10.69	-24.29
	0.4:1	-13.93	-28.67	-1.28	-29.35
HBSS	100:1	6.00	17.33	6.73	-4.50
	33:1	0.30	14.46	6.58	-9.50
	11:1	-10.32	9.65	-9.60	-5.68
	37:1	-14.34	1.66	-17.73	-13.69
	1.2:1	-23.70	-9.03	-30.78	-17.72
	0.4:1	-29.28	-12.56	-38.08	-17.75

* % lysis of ^{51}Cr labeled tumor cells
** NA = neuraminidase recombinant viral vector (rVV), MVA
= Ankara non-replicating vaccinia virus rVV, Kb = murine
H-2K^b MHC Class I molecule rVV, Ld = murine H-2L^d MHC
Class I molecule rVV, WB$_2$m = murine Beta 2-microglobulin
rVV, HBSS = Hank's Balanced Salt Solution

The secondary response of the mice is depicted in
Table 5. The results indicate a secondary immune response
seen by both VJS6 and B7.1 against the relevant target.

Table 5
Secondary Response of Mice 6 days After
I.V. Injection with Various Vaccinia Virus Constructs

rVV (pfu) Construct	Effector Target	Tumor cells			
		CT26	CT26/P	C25	CT26/V69
HBSS	100:1	30.26*	45.93	37.05	29.95
	33:1	15.83	13.38	10.05	18.24
	11:1	2.59	5.88	4.37	11.39
	37:1	0.83	5.64	-1.68	-1.00
	1.2:1	0.01	2.14	-2.71	4.12
	0.4:1	1.66	6.71	8.61	-0.11
VJS6 10^7	100:1	19.40	72.31	78.94	28.42
	33:1	21.52	83.69	63.82	18.04
	11:1	10.84	62.35	61.87	16.80
	37:1	7.91	44.31	52.99	4.75
	1.2:1	10.24	21.03	18.30	5.72
	0.1:1	4.59	14.14	11.92	10.07
VJS6 10^6	100:1	18.55	48.89	38.21	15.27
	33:1	11.33	41.52	34.52	8.25
	11:1	8.06	26.63	33.00	1.17
	37:1	2.57	16.54	29.26	-2.23
	1.2:1	7.86	14.96	13.57	0.56
	0.4:1	2.05	3.65	9.47	-8.17
Ld 10^7**	100:1	0.11	30.56	25.05	-4.91
	33:1	-2.78	25.03	26.24	-0.48
	11:1	2.49	16.01	11.97	-3.99
	37:1	-1.02	7.17	7.28	3.50
	1.2:1	0.52	9.57	4.36	4.06
	0.4:1	3.10	6.23	2.35	3.29
Ld 10^6	100:1	18.65	25.02	14.24	11.70
	33:1	0.63	11.23	7.51	11.36
	11:1	-0.35	7.47	10.24	2.76
	37:1	-0.47	1.38	-0.00	3.32
	1.2:1	-3.19	6.35	5.89	6.16
	0.4:1	4.08	5.70	2.86	4.04
B7.1 10^7	100:1	-10.62	83.64	69.56	-3.17
	33:1	0.20	63.61	59.63	0.23
	11:1	-6.10	40.58	61.77	3.02
	37:1	-1.91	22.29	22.86	-2.5
	1.2:1	-1.38	8.0	10.25	0.22

	0.4:1	4.73	8.39	9.49	2.22
B7.1 10^6	100:1	30.59	59.22	61.29	32.13
	33:1	31.24	74.4	53.99	23.29
	11:1	13.92	41.68	49.63	22.94
	37:1	10.66	31.02	34.86	5.63
	1.2:1	4.87	33.61	6.45	3.73
	0.4:1	8.78	7.66	3.98	7.16
MVA	100:1	28.16	18.13	25.09	30.30
	33:1	33.76	28.62	27.99	20.10
	11:1	28.69	12.63	9.75	9.13
	37:1	6.55	-0.43	12.79	2.78
	1.2:1	8.08	4.47	1.63	-0.45
	0.4:1	5.68	-1.30	1.21	4.60
MVA 10^7	100:1	22.77	32.85	28.05	27.07
	33:1	10.46	28.94	30.49	8.80
	11:1	5.48	24.20	22.32	-3.73
	37:1	0.49	11.9	12.82	4.36
	1.2:1	-0.95	5.29	13.32	6.36
	0.4:1	4.75	-0.73	14.69	9.59

* % lysis of ^{51}Cr labeled tumor cells
** NA = neuraminidase recombinant viral vecot (rVV), MVA = Ankara non-replicating vaccinia virus rVV, Kb = murine H-2K^b MHC Class I molecule rVV, Ld = murine H-2L^d MHC Class I molecule rVV, WB$_2$m = murine Beta 2-microglobulin rVV, HBSS = Hank's Balanced Salt Solution

Example 16

Clinical Protocol For Phase I/II Trials In Patients With Metastatic Melanoma and Metastatic Breast Cancer of Immunization with a Recombinant Vaccinia Virus Expressing the MAGE-1 Peptide and B7.1

In this protocol patients with advanced melanoma and advanced breast cancer are immunized against MAGE-1, an immunodominant peptide from a cancer antigen in combination with costimulatory/accessory molecule B7-1.

Patient Eligibility

Among other criteria, patients must have evidence of measurable or evaluable metastatic melanoma or breast cancer that has failed standard effective therapy. Patients must have tumors that express the MAGE-1 antigen

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as evidenced by PCR or Northern Blot analysis of tumor
cell RNA.

Treatment

Melanoma and breast cancer patients are divided into
two categories each, those who have previously been
vaccinated against smallpox and those who have not. Three
patients in each category receive intradermal immunization
with 10^8 plaque forming units/ml every four weeks for a
total of three doses. Patients are evaluated for
toxicity. When three patients in each category have been
followed for at least two weeks after the first
immunization without achieving grade 3 or 4 toxicity not
easily reversible by standard measures then the dose in
that plaque category will be escalated to 10^9 plaque
forming units/ml intradermally every four weeks for a
total of three doses. Fifteen patients in each category
will be treated at this dose and carefully evaluation of
toxicity, immunologic effects and therapeutic efficacy
will be evaluated.

If any patient receiving the 10^8 pfu/ml dose achieves
grade 3 or 4 toxicity not easily reversible by standard
measures, then an additional three patients will be
treated at that dose. If a second patient develops grade
3 or 4 toxicity, not easily managed by standard
procedures, then the dose will not be escalated in that
category.

Vaccine preparation. The recombinant vaccinia virus
used in this protocol is the Wyeth vaccinia virus derived
from the New York City Board of Health strain. This is an
attenuated virus with an extensive history of previous use
in humans. The recombinant virus has the gene coding for
the MAGE-1 nine amino acid minimal determinant plus the
B7.1 sequence inserted into the viral thymidine kinase
gene.

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The recombinant virus is produced in a Food and Drug Administration approved facility for the manufacture of GMP grade clinical material.

Vaccination procedure. The recombinant vaccinia virus will be supplied as a sterile, frozen suspension and will be approximately diluted to either 10^8 or 10^9 plaque forming units/ml with phosphate buffered saline.

Vaccination will be performed in the deltoid muscle area. The method of vaccination is summarized as follows:

a) Open the bifurcated needle by catching the butt-end of the needle and gently pulling the point end free.

b) Dip the bifurcated point of the needle into vaccine. The needle will pick up a drop of vaccine in the space between the two points.

c) Using the same bifurcated needle, use the multiple pressure technique by pressing the needle through the vaccine drop on the subject's skin. Fifteen needle pressures will be administered.

d) Alternative upper extremities will be used for subsequent vaccinations.

Patients will be observed for 24 hours after immunization. Body temperature will be measured at 12 hours and at 24 hours and any adverse reactions noted.

Post vaccination evaluation

On day 14 after vaccination, patients will be seen and the following tests obtained:

a) Complete history and physical examination, including any symptoms and progress of reaction from the vaccination.

b) Photograph of the vaccination site.

c) Complete blood count with differential count.

d) Platelet counts.

e) Acute care, hepatic, mineral and thyroid blood chemistry panel.

f) Serum and lymphocytes stored as performed

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pretreatment.

Patients will be evaluated at 14 days following the second vaccination and similar procedures will be performed prior to and following the third and final vaccination on day 56.

Patients will have a complete restaging of all sites of disease with appropriate physical examination and x-rays and nuclear medicine studies at the time of one and two months following the final vaccination.

Immunologic studies

Immunologic assessment will be made of the patient's response to vaccinia antigens as well as to the MAGE-1 antigen.

a) All serum samples will be tested for anti-vaccinia antibody by ELISA.

b) All cryopreserved lymphocytes will be tested for response to MAGE-1 antigen using limiting dilution analysis of precursor CTL frequency using the method of Coulie, P et al International Journal of Cancer 50:289-297, 1992.

c) Patients with easily accessible disease may have biopsy under local anesthesia of accessible tumor to study the histopathologic nature of the tumor as well as the isolation of tumor infiltrating lymphocytes for in vitro growth. Tumor infiltrating lymphocytes will be tested for specific reactivity and specific cytokine release against MAGE associated antigens.

Assessment of response

A complete response is defined as the disappearance of all clinical evidence of disease that lasts at least four weeks.

A partial response is a 50% or greater decrease in the sum of the products of the perpendicular diameter of all measurable lesions for at least four weeks with no appearance of new lesions or increase in any lesions.

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Minor responses are defined as 25-49% decrease in the sum of the products of the perpendicular diameters of all measurable lesions with no appearance of new lesions and no increase in any lesions.

Any patient with less than a partial response will be considered a non-responder.

The appearance of new lesions or greater than 25% increase in the product of perpendicular diameters of prior lesions following a partial or complete response will be considered as a relapse.

Similar protocols will be followed for the evaluation of other recombinant virus vaccines against cancer. Vaccines to be tested include but are not limited to Recombinant Vaccinia virus encoding MART-1 and IL-2, Recombinant Vaccinia virus encoding MART-1 and B7.1, Recombinant Vaccinia virus encoding GP100 and IL-2, Recombinant vaccinia virus encoding GP100 and B7.1, Recombinant fowlpox virus encoding MART-1 and IL2, recombinant fowlpox virus encoding MART and B7.1 and the like with the appropriate modifications depending on the antigen and virus used.

Example 17

Use of Lymphocytes Sensitized To Immunogenic Peptides Derived From Melanoma Antigens For Therapeutically Treating Mammals Afflicted With Melanoma

T-lymphocytes presensitized to the melanoma antigen may be effective in therapeutically treating mammals afflicted with melanoma. T-lymphocytes are isolated from peripheral blood or melanoma tumor suspensions and cultured in vitro (Kawakami, Y. et al. (1988) J. Exp. Med. 168:2183-2191). The T-lymphocytes are exposed to cells infected with the recombinant virus expressing a melanoma associated antigen and IL2 and/or B7.1 for a period of about to 1-16 hours at a concentration of 1 to 10mg/ml. T-lymphocytes exposed to the antigen will be administered

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to the mammal, preferably a human at about 10^9-10^{12}
lymphocytes per mammal. The lymphocytes may be
administered either intravenously, intraperitoneally or
intralesionally. This treatment may be administered
concurrently with other therapeutic treatments such as
cytokines, radiotherapy, surgical excision of melanoma
lesions and chemotherapeutic drugs, adoptive T lymphocyte
therapy.

Example 18

Active Immunotherapy with Recombinant Vaccinia Virus Co-expressing B7-1, B7-2 or Both and a Model Tumor Antigen Mediates Regression of Established Pulmonary Metastases

Materials and Methods

Animals

Female BALB/c (H-2^d) mice were obtained from
Frederick Cancer Research Center (Frederick, MD). All
mice were used at 6 - 8 weeks of age.

Preparation of Cell Lines

CT26 is an N-nitroso-N-methylurethrane induced BALB/c
(H-2^d) undifferentiated colon carcinoma as supplied by D.
Pardoll (Baltimore, MD). CT26 was cloned to produce a
wild type parental tumor line, CT26.WT. The gene for *lac*
Z was stably transfected into CT26.WT as previously
described herein and in Wang, M. et al *J. Immunol.*
154(9):4685-4692, 1995. Briefly, a plasmid donated by
A.D. Miller containing the gene for β-galactosidase and a
neomycin resistance marker were used to construct the LZSN
amphotropic retrovirus which was used to transduce
CT26.WT. Transductants were selected in G418 media, and
subcloned by limiting dilution analysis at 0.3 cells/well.
Subclones that expressed β-galactosidase were screened by
X-gal staining and in ^{51}Cr release assays with anti-β-
galactosidase effectors. The subclone CT26.CL25 was
selected for use in all studies because of its stable
expression of both β-galactosidase and the class I

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molecule H-2 L^d. BS-C-1 or HeLa S^3 cells (ATCC, American
Tissue Typing Collection, Rockville, MD CCL 16) were used
to expand and titer all viruses. Cell lines were
maintained in RPMI 1640, 10% heat inactivated FCS
(Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml
streptomycin, 100 μg/ml penicillin and 50 μg/ml gentamicin
sulfate (NIH Media Center). CT26.CL25 was maintained in
media containing 400 or 800 μg/ml G418 (GIBCO, Grand
Island, N.Y.).

**Construction and Characterization of Recombinant Vaccinia
Viruses**

       Construction and characterization of recombinant
vaccinia viruses containing murine B7-1(v.MCB7-1), B7-2
(v. MCB7-2), B7-1 and B7-2 (v.B7-1/B7-2) and measles
hemagglutinin (HA) (v.MCMHA) is as follows. Briefly, the
B7-1 gene (a gift from Dr. R. Germain, NIH) and the
measles HA gene (a gift from Dr. S. Rozenblatt, Tel Aviv
University, Israel) were cloned into the transfer plasmid
pRB12 such that they were under control of the VV
synthetic early/late promoter. The gene of interest was
inserted into the Hind III F region of VV. Methods used
for the production and selection of rVV in which the VV
thymidine kinase gene was utilized as an insertion site,
were similar to those described previously by Earl, P.L.
et al. In: Current Protocols in Molecular Biol. Ausubil
et al (eds) Greene Publishing Assoc. and Wiley
Interscience, 1991, 16.18.1-16.18.10 unless otherwise
stated.   The v.MCB7-1/β-gal and v.MCMHA/β-gal were
constructed by homologous recombination of the plasmid
pSC65Δ (a modification of pSC65 in which the E. coli *lac Z*
gene is under transcriptional control of the early
function of the VV p 7.5k promoter and flanked by VV
derived TK DNA) with v.MCB7-1 and v.MCMHA respectively.
The v. MCB7-2/β-gal was constructed with both the B7-2
gene and the E. coli *lac Z* gene under transcriptional

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control of the early function of the VV p 7.5k promoter
and flanked by VV derived TK DNA. The v. MCB7-1/B7-2/β-
gal was constructed by homologous recombination of the
plasmid pSC65Δ (modified as stated previously to create
the v. MCB7-2/β-gal construct) with the v. MCB7-1.

Table 6.   Construction of recombinant vaccinia viruses.

Recombinant Vaccinia Virus Code	VP37		Thymidine Kinase	
	Promoter	Gene	Promoter	Gene
v.MCB7-1/β-gal	S.E/L	B7-1	7.5e	β-gal
v.MCMHA/β-gal	S.E/L	MHA	7.5.e	β-gal
v.MCB7-1/NP	S.E/L	B7-1	7.5e	NP
v. MCB7-2/β-gal			7.5e	B7-2, β-gal
v. MCB7-1/B7-2/β-gal	S.E/L	B7-2	7.5e	B7-2, β-gal
v.MCMHA/B7-2/B-gal	S.E/L	MHA	7.5e	B7-2, β-gal

MHA= measle hemagglutinin gene, B7-1 = murine B7-1, B7-2 =
murine B7-2, NP = influenza nucleoprotein, β-gal= E.coli
β-galactosidase gene, S.E/L = vaccinia synthetic early
late promoter, 7.5e = early only function of the vaccinia
7.5k promoter.

Recombinant viruses were simultaneously selected for
their TK negative phenotype and ability to express β-
galactosidase. Transfer plasmid pGS69 (Smith, G.L. et al
Virology 160:336-345, 1987 containing the influenza
nucleoprotein (NP) was recombined with v.MCB7-1 to yield
v. MCB7-1/NP. This recombinant virus was selected on the
basis of a TK negative phenotype. Plaques were then
analyzed for expression of NP by immunostaining (Sutter,
G. et al Vaccine 12:1032-1040, 1994) and subsequently
plaque purified under agar overlay four times before
sucrose cushion purified viral stocks were prepared (Earl,
P.L. et al Current Protocols in Mol. Biol., ibid (Table

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6.).

Preparation of rVV expressing the influenza A/PR/8/34
nucleoprotein (NP) was previously described (Smith, G.L.
et al *Virology* 160:336-345, 1987). In the control
construct, v. JS6, the E.coli *lac Z* gene was under the
control of the early VV p 7.5k promoter from plasmid
pSC65. Murine IL-2 cDNA was amplified by polymerase chain
reaction from pBMGNeomIL-2 and ligated into the *Sma I-
BamHI* site of a vaccinia expression vector, pMJ601, which
contains the b-galactosidase gene under the control of VV
early p 7.5k promoter. The other cytokines (GM-CSF, IFN-
g, and TNF-a) were inserted into the wild type VV genome
with similar procedure. The techniques used in preparation
of the v. HLA.A2.1/β-gal (O'Neil, B.H. et al *J. Immunol.*
151:1410-1418, 1993) v. human and murine β_2-
microglobulin/β-gal, (O'Neil, B.H. et al *J. Immunol.*
151:1410-1418, 1993) and all the murine MHC I (L^d, D^d, K^d,
and K^b)/β-gal (Restifo, N.P. et al *J. Exp. Med.* 177:265-
272, 1993), has been previously described.

Analysis of Recombinant Proteins and Quantitation of β-galactosidase Expression

BS-C-1 monolayers were infected with 10 infectious
units of recombinant virus per cell. After 20 hours
infected cells were harvested in solubilization buffer
(0.06M Tris-HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol,
10% glycerol and 0.002% bromophenol blue). Proteins were
resolved on a 10% polyacrylamide gel and subsequently
transferred to a nitrocellulose membrane using a Bio-Rad
Transblott Mini cell (Bio-Rad) at 250 mAmps for 1 hour.
Membranes were initially incubated for 1 hour in a
blocking solution (PBS-B) consisting of PBS containing 3%
non-fat milk (w/v) and 0.2% Tween 20. A murine specific
B7-1 hamster antibody (supplied by Dr. H. Reiser, Dana-
Farber Cancer Inst, Boston) was diluted in PBS-B and
incubated with filters for 3 hours at room temperature.

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Filters were subsequently washed three times in PBS
containing 0.2% Tween 20. Bound primary antibodies were
detected by incubating filters, after washing with ^{125}I-
protein A (Amersham) at a concentration of 0.1μCi/ml in
PBS-B. Filters were washed 4 times dried and exposed to
X-ray film (Kodak). Protein sizes were estimated using 14C
molecular weight markers (Amersham) (Fig. 13).

Recombinant viruses were analyzed for β-galactosidase
expression using an enzyme assay kit (Promega, WI).
Briefly, monolayers of BS-C-1 cells were infected at a
multiplicity of infection (MOI) of 10. Approximately 20
hours post infection cells were washed with PBS and
harvested with lysis buffer. Quantitation of enzyme
expression was determined by incubating cell extracts with
β-galactosidase substrate and buffer solution as described
in the manufactureres protocol.

In Vivo Experiments

In vivo protection studies utilized BALB/c mice which
were immunized intravenously with either Hanks's balanced
salt solution (HBSS) (Biofluids, Inc., Rockville, MD),
v.MCB7-1/b-gal, or v.MCMHA/b-gal (10^{7}plaque forming units
(PFU)). Twenty-one days following this initial
immunization, the mice were intravenously challenged with
5 x 10^5 tumor cells of CT26.WT or CT26.C25. Twelve days
following tumor challenge, mice were randomized,
euthanized, and lung metastases were enumerated in a
blinded fashion.

In vivo adoptive transfer studies involved
intravenous immunization of BALB/c mice with either HBSS,
v.MCB7-1/b-gal or v.MC MHA/b-gal . The rVV was
administered at either 10^5 or 10^7 PFUs. After twenty-one
days, these mice were euthanized, and splenectomized.
Following splenectomy, the spleens were morselized, passed
through a Nytex membrane, and suspended in HBSS.
Lymphocytes were counted on a hemocytometer, and 2 x 10^7
lymphocytes were adoptively transferred to mice who had

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been injected intravenously with 5 x 10⁵ tumor cells of
either CT26.WT or CT26.C25. Designated groups of mice
received adjuvant treatment with six doses of IL-2
administered intra-peritoneal at a dose of 100,000 Cetus
units two times per day for three days. Nine days after
adoptive transfer of primed splenocytes, the mice were
randomized, euthanized, and lung metastases counted in a
blinded fashion.

    In vivo active treatment studies involved non-
irradiated BALB/c mice which were inoculated
intravenously with 5 x 10⁵ tumor cells of either CT26.WT
or CT26.C25. All mice were randomized and subsequently
vaccinated with HBSS, or 10⁵ or 10⁷ PFUs of the designated
rVV three or six days later. Designated groups of mice
received adjuvant treatment with six doses of IL-2
administered intra-peritoneal at 100,000 Cetus units two
times per day for three days. Mice were randomized and
euthanized on day twelve, and lung metastases were counted
in a blinded fashion. Identically treated groups of mice
bearing three day tumor burdens were also followed long
term to assess the effect of vaccination on survival.

T cell Subset Depletions

    Purified culture supernatants of anti-CD4 monoclonal
antibody GK1.5 (TIB 207; American Tissue Culture
Collection) and ascitic fluid of hybridoma 2.43 (anti-CD8)
(TIB 210: American Tissue Culture Collection) were diluted
in HBSS prior to use in vivo. For in vivo depletion
BALB/c mice were given i.v. injections of GK1.5 at 100
mg/ml or of empirically determined levels of 2.43
monoclonal antibodies 48 hours prior to receiving tumor
challenge, and again 6 days later. Using fluorescein
isothiocyante-labeled anti-CD4 and phycoerythrin labeled
anti-CD8 antibodies, FACS analysis was performed 1 day
prior to immunization, and again at day 7 to verify
depletion.

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Whole Organ X-gal Staining

Lungs from tumor-bearing mice were removed and
inflated with PBS (Biofluids) prior to X-gal staining.
Lungs were fixed in a solution containing 2% formaldehyde
(v/v), 0.2% gluteraldehyde (v/v) in PBS for 45 minutes and
washed with PBS three times. After washing, the lungs
were stained with X-gal solution for 12 hours at 37^0 C.
X-gal solution for whole organs was prepared by combining
the following: 0.02% (v/v) NP-40, 0.01% (w/v) sodium
deoxycolate, 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 2
mM MgC12 in PBS. After staining in X-gal solution, the
lungs were rinsed briefly with 3% (v/v) dimethyl sulfate,
and then with PBS and stored at 4^0 C in 0.02% (w/v) sodium
azide in PBS.

Statistical Analysis

Data from *in vivo* protection, adoptive, and active
treatment studies was analyzed using a two-sided
student's *t* test for unpaired samples. Errors bars
represent the standard deviation within each group.
Survival was analyzed with standard Kaplan-Meier survival
curves (Kaplan, E.L. J. Am. Stat. Assoc 53:457-481, 1958).
All p values presented are two-sided.

Example 19

Characterization of Murine B7-1 and E.coli
β-gal Genes in Cells Infected by Vaccinia Viruses

Following preparation of the rVVs, and prior to their
use in *in vivo* experiments, the the *in vitro* expression of
foreign protein in infected cells was evaluated by either
immunohistochemical staining, western blot analysis, or
both. Western blot analysis of murine B7-1 expression of
virally infected cells illustrated the previously
reported diffuse staining pattern typical of highly
glycosylated proteins (Fig. 13) (Freeman, G.J. et al J.
Exp. Med. 174:625-631, 1991). Molecular weight markers
estimate VV expressed B7-1 to be approximately 55-65 k Da

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in size. The authenticity of rVV expressed murine B7-1
was further verified by the binding of CTLA4-Ig to v.
MCB7-1 infected cells. Expression of rVV murine B7-2 was
verified by immunohistochemical staining of infected cells
using a murine specific B7-2 antibody (PharMingen, CA).
5   Expression of the TAA, β-galactosidase, was similar for v.
MCB7-1/β-gal and the control recombinant v. MCMHA/β-gal,
in contrast v. MCB7-1/NP showed no signs of enzyme
expression (data not shown).

10   Example 20

                 Initial Screen: Ability of Immunization with
                 Recombinant Vaccinia Virus to Mediate Regression
                             of Established Tumor

         It was determined whether combining a TAA with one or
15   several of these immunomodulatory molecules would enhance
     the antigen specific immune response, as measured by the
     most stringent test, i.e., the ability to mediate
     regression of established disease.   The virus, v. JS6,
     expressing the TAA alone, was used as control to measure
20   either the positive or negative contribution of the
     immunomodulatory molecule in the vaccination vector.
         The ability of a single inoculation of twelve double
     rVVs, which expressed the genes encoding both an
     immunomodulatory molecule and the β-gal model tumor
25   antigen in the same virus, to effect regression of
     pulmonary metastases in tumor-bearing mice was assayed.
     The double rVVs were constructed to include the b-
     galactosidase gene within the thymidine kinase (TK) region
     of the VV genome for all vectors evaluated.   The position
30   of the immunomodulatory molecule for most vectors is also
     within the TK region of the VV genome under the same
     promoter as the TAA. However, in the case of the B7-1
     vector, the gene for B7-1 is placed within the Hind III F
     region as described above and shown in Table 6.
35   The double rVVs studied were v.MCB7.1 (Hind III F

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region of VV genome)/b-gal (TK region of VV genome), v.
human b₂-microgulin (TK)/b-gal(TK) {3154}, v. murine b₂-
microgulin (TK)/b-gal(TK) (O'Neil, B.H. et al J.
Immunol. 154:1410-1418, 1993), v. murine MHC I L^d (TK)/b-
gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993),
v. MHC I K^d (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med.
177:265-272, 1993), v. MHC I D^d (TK)/b-gal (TK), v. MHC I
(allogeneic) K^b (TK)/b-gal (TK) (Restifo, N.P. J. Exp.
Med. 177:265-272, 1993), v. human leukocyte antigen
(xenogeneic) HLA-A₂ (TK)/b-gal (TK) (O'Neil, B.H. et al
J. Immunol. 154:1410-1418, 1993), v. murine interleukin -
2 (IL-2) (TK)/b-gal (TK), v. murine granulocyte-macrophage
colony stimulating factor (GMCSF) (TK)/b-gal (TK), v.
interferon-gamma (IFN-g) (TK)/b-gal (TK) (Bronte et al.,
in press), and v. tumor necrosis factor-alpha (TNF-a)
(TK)/b-gal (TK). The v. JS6, expressing only b-gal in the
TK region (described above), and a non-replicating VV,
modified vaccinia Ankara (MVA) (Sutter, G. et al Proc.
Nat'l Acad. Sci. U.S.A. 89:10847-10851, 1992), also only
expressing b-gal, were used as controls.

Using the *in vivo* active treatment protocol outlined
above, mice bearing three day pulmonary metastases were
immunized with a single dose of the selected rVV. In each
experiment, no therapeutic response was seen with any rVV
against the parental tumor, CT26.WT, thus documenting the
specificity of the response. Figure 14 displays the
results of our initial screening experiment in which
mice bearing three-day CT26.C25 tumors were vaccinated
with rVV (10^7 PFUs).

When compared with the HBSS vaccinated control mice,
only v. IL-2/b-gal and v.MCB7-1/b-gal mediated both a
specific and significant reduction in the number of
pulmonary metastases (p < 0.006 and p< 0.002,
respectively). The ability of v. JS6, the single rVV
expressing b-gal alone, to mediate significant tumor
regression was variable from experiment to experiment.

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(Data not shown). The inability of v. MVA/b-gal, a non-replicating VV which produces high levels of b-gal, to mediate any regression, may indicate that prolonged TAA expression (as would occur with a replicating VV) is required for a successful CTL response.   Treatment with v. IL-2/b-gal and v. MCB7-1/b-gal resulted in a 10 and 5 fold further reduction in the number of metastases when compared to the best results obtained with v. JS6. These results suggest that vaccination with an immunodominant TAA alone is capable of priming a cytotoxic antitumor immune response which can then be further enhanced by the simultaneous expression of unique immunomodulatory molecules.

Although all double rVV contained the gene for the TAA, b-gal, only v. IL-2/b-gal and v. MCB7-1/b-gal were able to mediate significant reductions in the number of pulmonary metastases. Insufficient b-gal expression is unlikely to account for the therapeutic failure of the ineffective double rVVs. When tested, b-gal expression, at least between the individual cytokine producing rVV, did not significantly different (data not shown). The potential for the gene encoding for the immunomodulatory molecule within the double rVV to exert an immunosuppressive effect on the generation of a TAA cytotoxic response, or directly inhibit TAA protein expression was not evaluated, and cannot be excluded. The possibility that the failure of the GM-CSF, IFN-γ, or TNF-α producing rVVs is due to immunosuppresive effects of local cytokine production was not further explored.

Tumor regression following vaccination with v. MCB7-1/b-gal has not been previously reported.   However, the therapeutic result obtained with this rVV supports the notion that costimulatory molecule expression is necessary for optimal T cell activation.   Of note, a single vaccination with v. D^4/b-gal at a lower viral inoculate (10^6 PFUs) was also capable of mediating a

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significant reduction in the number of pulmonary
metastases (p < 0.005). This suggests the existence of a
D^d epitope for the b-gal protein heretofore unknown.

Based on the findings of this screen, and other
reports of anti-tumor effects with both B7-1 transfectants
(Guinan, E.C. Blood 84:3261-3282, 1994) and v. B7-1
oncolysates (Hodge, J.W. et al Cancer Res. 54:5552-5555,
1994), a more extensively evaluate the in vivo effects of
v. MCB7-1/b-gal on the cell-mediated antitumor immune
response was conducted. Interestingly, the generation of
CTL following B7-1 transfection is an IL-2 dependent
phenomenon, since the addition of IL-2 blocking antibody
during the induction phase blunts generation of a
cytotoxic response (Harding, F.A. et al J. Exp. Med.
177:1791-1796, 1993).

Example 21

Protection Against Subsequent Tumor Challenge
by Immunization with v. MCB7-1/β-gal

Although the identification of murine and human TAAs
and TAA specific CTLs confirm that an immunocompetent
host can generate a significant cytotoxic T cell response
against autologous neoplasms; the immunogenicity of these
TAA is generally weak allowing for the progressive growth
of tumors. Protective immunity following vaccination with
a rVV expressing either the TAA alone, or in combination
with B7-1 was evaluated. The rVV strategy utilized in
this current study has a distinct advantage over B7
transfections studies (in addition to the ease of
administration), since the specificity of the immune
response against a defined model TAA can be measured by
comparing the response in the transduced tumor cell line
CT26.C25, with the parental tumor, CT26.WT. Whereas the
protective immunity demonstrated in the transfection
studies is interpreted as a specific CTL response against
an undefined TAA, the unique reactivity of the CTL in this
model is easily evaluable.

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Naive mice were initially vaccinated with either v.
MCB7-1/b-gal, or a control virus, v. MCMHA/b-gal. The v.
MCMHA/b-gal vector expressed an irrelevant protein,
measles HA, which is a similarly sized and glycosylated
molecule like B7-1, as well as the model TAA to allow
for evaluation of the contribution of B7-1 to the response
generated. Mice immunized with both v. MCB7-1/b-gal and
v. MCMHA/b-gal were protected against tumor challenge
with CT26.CL25, but not the wild type tumor, CT26.WT (p <
0.0002 and p < 0.0014, respectively) (Fig. 15.). These
results confirm previous reports that vaccination with a
rVV expressing a unique TAA alone can confer protection
to subsequent challenge with a TAA-expressing tumor.
(Kantor, J. et al J. Nat'l Cancer Inst. 84:1084-1091,
1992; Estin, C.D. et al Proc. Nat'l Acad. Sci. U.S.A.
85:1052-1056, 1988). The completeness of the protection
by both viruses precluded a more thorough evaluation of
the impact of the B7-1 gene in this situation.   In this
model system, b-gal protein expression by both v. MCMHA/b-
gal and v. MCB7-1/b-gal was sufficient to exploit
intracellular antigen processing mechanisms leading to
protective immunity.

Example 22

Primary Adoptive Transfer of v. MCB7-1/β-gal
Primed Splenocytes to Tumor-Bearing Syngeneic Mice

Tumorigenicity is reflective of the state of T cell
activation. In the present invention it was hypothesized
that vaccination a rVV coexpressing B7-1 and a model TAA,
would result in the generation of a primary TAA CTL
response which could be used in the adoptive therapy of
tumor-bearing syngeneic host. The therapeutic advantage
gained by immunizing against a known TAA to elicit CTLs
reactive against both autologous or allogeneic tumors are
numerous. Confirmation of the specificity of the CTL
response was demonstrated in the initial observation that
no reduction in the number of pulmonary metastasis was

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noted after the adoptive transfer of any group of primed
lymphocytes to syngeneic mice bearing CT26.WT tumors.

Mice bearing CT26.C25 tumors and inoculated with HBSS
primed splenocytes showed no therapeutic benefit, however
the addition of adjuvant IL-2 to similarly treated mice
resulted in a significant reduction in the number of
pulmonary metastases (p <.0054) (Fig. 16.).   CT26.C25
tumor-bearing mice treated with v. MCB7-1/b-gal rVV
splenocytes at two doses (10^5 and 10^7 PFUS) showed a
significant reduction in tumor burden (p < 0.007 and p <
0.007, respectively). Again, adjuvant IL-2 treatment
resulted in a 16.5 and 12.5 fold further reduction in the
number of pulmonary metastases in this group.
Significant tumor regression was also seen in mice
receiving splenocytes primed with v. MCMHA/b-gal at 10^5
PFU, but not at 10^7 PFU (p < 0.0052, and p < 0.094,
respectively).   The addition of adjuvant IL-2 to the v.
MCMHA/b-gal treatment group resulted in a 2 fold reduction
in the number of pulmonary metastases in the group treated
with v. MCMHA//B-gal primed splenocytes at 10^5 PFU.

Vaccination with a rVV expressing only the model TAA,
was capable of priming a specific immune response which
mediated tumor regression on adoptive transfer.
Vaccination with a rVV containing both the costimulatory
molecule, B7-1, and the model TAA, increased the
therapeutic response by up to 150 fold.   The increased
therapeutic benefit mediated by v. MCB7-/b-gal primed
lymphocytes may represent either or both a quantitative
increase in the precursor frequency of TAA specific CTL in
the adoptively transferred population, or a qualitative
increase in the cytotoxic potential of the transferred
cells mediated by B7/CD28 costimulatory signals.
Infection with v. MCB7-1/b-gal would provide any MHC class
I expressing cell the required elements necessary to
mediate a CTL response, without the need for APCs or CD4+
T helper cells (Dohring, C. et al _Int. J. Cancer_ 57:754-

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759, 1994; Wu, Y. et al *Curr. Biol.* 4:499-505, 1994).
Infection with v. MCB7-1/b-gal would also provide T cells
with the capacity for autonomous stimulation leading to
both a proliferative response to antigen and increased
cytokine production (Dohring, C. et al ibid; Azuma, M. et
al *J. Exp. Med.* 177:845-850, 1993). Notably, binding of
B7/CD28 costimulates IL-2 mRNA accumulation (Linsley,
P.S. et al *J. Exp. Med.* 173:721-730, 1991) important for T
cells proliferative response, as well as IFN-γ, important
in Th1 induction, as well as IL-4 important in Th2
induction (Walter, H. et al. *Eur. Cytokine Netw.* 5:13-21,
1994). In the absence of an antigen non-specific
costimulatory signal delivered by interaction between B7-
CD28, naive T cells exhibit suboptimal proliferation and
decreased stability of mRNA for several cytokines
important to the T cell proliferative and differentiation
response (Linsley, P.S. et al *Annu. Rev. Immunol.* 11:191-
212, 1993; Harding, F.A. et al *J. Exp. Med.* 177:1791-1796,
1993). Coculture of CD8+ T cells with B7+, but not B7-
tumors results in potent cytotoxic response, and
proliferation (Harding, F.A. et al *J. Exp. Med.* 177:1791-
1796, 1993), which are inhibited by both anti-CD28 Fab
fragments, and interestingly by anti-IL-2 antibodies. The
results demonstrate that direct activation of CTL by rVV
which provide appropriate costimulation and antigen
specificity results in improved therapeutic response upon
the adoptive immunotherapy of primed splenocytes to
syngeneic tumor-bearing mice.   The mechanism responsible
for this response may be due to either or both a
qualitative increase in antigen specific T cell precursors
mediated by B7/CD28 (or CTLA-4) interaction, or B7/CD28
(or CTLA-4) mediated differentiation of T cell effector
populations.

     The adjuvant benefit received by adjuvant IL-2 alone
upon the adoptive transfer of splenocytes in these studies
confirms prior reports documenting direct IL-2 antitumor

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responsiveness. The simplest explanation for the
therapeutic response seen with IL-2 alone is that is
represents a lymphokine activated killer cell (LAK)
response mediating non-specific cytotoxicity. However,
IL-2 treatment was therapeutic only in the mice bearing
CT26.C25 tumors, which expressed the model TAA.
Therefore, a more likely explanation for results seen
with IL-2 alone and as an adjuvant, is an IL-2 mediated
proliferation of antigen-specific CTL.

Example 23

Active Immunotherapy of Established Pulmonary Metastases with v. MCB7-1/β-gal

Treatment of established disease remains the ultimate
goal of immunotherapy. If lack of costimulation is a
principal means through which tumors evade immune
surveillance in this model system, then vaccination with a
v. MCB7-1/b-gal should generate an active and specific
therapeutic immune response in animals bearing established
tumor. To test this hypothesis, tumor-bearing mice were
immunized with a single injection of rVVs, with designated
groups receiving adjuvant treatment with IL-2 as outlined
above. In addition to v. MCB7-1/b-gal and v. MCMHA/b-gal,
v. MCB7-1/NP was used in these studies. The v. MCB7-1/NP
co-expresses the B7-1 protein with an irrelevant protein
(influenza nucleoprotein, NP) not expressed on the
CT26.C25, and allows for assessment of the role of B7-1
expression alone. No significant tumor regression was
seen following rVV vaccination in mice bearing CT26.WT
tumors.

In mice bearing CT26.C25 tumors, immunization with v.
MCB7-1/b-gal mediated a significant reduction in the
number of metastases ($p < 0.006$) (Fig. 17.). The high
therapeutic effect precluded evaluation of the effect of
adjuvant IL-2. In the same experiment, mice immunized
with v. MCMHA/b-gal mediated no significant tumor
regression ($p < 0.36$). In four separate experiments a

significant decrease in the number of pulmonary metastases
for mice immunized with v. MCMHA/b-gal at 10^7 PFU was seen
only once (p < 0.009). IL-2 administration did not
significantly improve upon tumor regression in v. MCMHA/b-
gal treated mice. In the same and subsequent experiments,
v. MCB7-1/NP had no effect on tumor regression. Overall,
mice immunized with v. MCB7-1/b-gal showed a 170 fold and
117 fold greater reduction in the number of pulmonary
metastases than mice in the v. MCMHA/b-gal and v. MCB7-
1/NP immunization groups, respectively. Exogenous IL-2
alone did not mediate further tumor regression. This data
implies that expression of the TAA and the costimulatory
molecule are both necessary and sufficient to effect
maximal regression of tumor in this active treatment model
and suggests a role for B7-1 in both the induction and
effector phase of the CTL response. This conclusion is
further supported by the finding that a vaccination vector
combining b-gal with an irrelevant protein did not
reliably reduce tumor burden. (Note in one of four
experiments, a significant reduction was observed,
although to a much lesser degree than that seen in the v.
MCB7-1/b-gal immunization group.) The presence of B7-1
alone was insufficient to effect any significant
regression, which is consistent with reports that the mere
expression of B7-1 on tissue without a new or foreign
antigen does not result in tissue specific autoimmunity
(Guerder, S. et al _Immunity_ 1:155-166, 1994; Harlan, D.M.
et al _Proc. Nat'l Acad. Sci._ U.S.A. 91:3137-3141, 1994).

      The results from four separate active immunotherapy
studies of mice immunized with 10^7 PFUs of rVV were
normalized to the HBSS group for each individual
experiment, and the data pooled and analyzed as percent
residual disease (data not shown). Immunization with v.
MCB7-1/b-gal group showed an almost 20 fold reduction in
the tumor burden compared to HBSS control, with only 5.2%
of tumor remaining. Adjuvant IL-2 treatment did not

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improve upon this result, though a trend was seen in this
group and the v. MCMHA/b-gal group. Immunization with v.
MCMHA/b-gal mediated a nearly 2 fold reduction in the
number of metastases with 40-55% of tumor burden
remaining, while the v. MCB7-1/NP mediated no meaningful
reduction with 83-93% of tumor burden remaining. This
cumulative data confirms the finding seen in the
individual active immunotherapy studies.

     In order to evaluate whether active immunotherapy
results with v. MCB7-1/b-gal were limited to the treatment
of small tumor burdens, the same active treatment
experiment in mice bearing six day metastases as repeated.
 A six day tumor burden is macroscopically visible with
individual metastases measuring up to 2.5 millimeters.
Again, no significant tumor regression was seen following
rVV vaccination in mice bearing CT26.WT tumors. Figure 18
demonstrates that in the six day model, a single
immunization with v. MCB7-1/b-gal mediated a significant
reduction in the number of pulmonary metastases in mice
bearing CT26.25 tumors (p < 0.008). Mice immunized with
either v. MCMHA/b-gal or v. MCB7-1/NP had no significant
reduction in the number of pulmonary metastases (p < 0.12,
and p < 0.22, respectively). Again, adjuvant IL-2 did not
improve this result. It is concluded therefore, that a
rVV co-expressing both the costimulatory molecule, B7-1,
and the TAA, b-gal, are required to mediate regression of
both large and small tumor burdens in this model system.
Consistent with the previous data disclosed herein and in
Wang et al J. Immunol. 154(9): 4685-4692, 1995,
immunization with a rVV containing only the immunodominant
TAA, b-gal, was capable of inducing a cytotoxic immune
response resulting in a reduction in the number of
pulmonary metastases when small tumor burdens are present,
but has no efficacy against a larger tumor burden.
Immunization with a rVV expressing the costimulatory
molecule alone without the TAA, i.e., v. MCB7-1/NP, was

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unable to prime an immune response and mediate tumor regression in either situation.

A reduction in tumor burden is frequently demonstrated for several adjuvant interventions, but rarely translates into a therapeutic benefit in terms of prolonged survival. In a parallel experiment to the three day active treatment protocol described above, ten mice per group were followed daily for survival and events were recorded as either deaths, or severely moribund condition requiring euthanasia. In this study, all mice inoculated with CT26.WT were dead by day 43 with 50% dead in all groups by day 32.

CT26.C25 tumor bearing mice demonstrated varying degrees of survival among vaccination groups (Figure 19). Mice receiving HBSS, IL-2 alone, and v. MCB7-1/NP alone (with or without adjuvant IL-2), were dead by day 39. Mice vaccinated with v. MCMHA/b-gal showed a significant prolongation of survival with 50% of mice alive at day 27, and the last mouse dying at day # 46 (p < 0.0002) Adjuvant IL-2 did not statistically improve upon this result. In the v. MCB7-1/b-gal vaccinated mice 50 % were alive at 70 days, and 30% continue to be long term survivors at over 100 days (p < 0.0001). Adjuvant IL-2 therapy for this group added no statistically significant benefit to survival, though a trend was suggested with 50% still alive at day # 88, and 40% alive at 100 days follow-up.

The regression of pulmonary metastases seen following immunization with the v. MCB7-1/ b-gal thus translated into prolonged survival for most animals treated. Two mice who were long-term survivors in the v. MCB7-1/b-gal treated group, subsequently died and their lungs were subjected to whole organ X-gal staining. One of the mice showed no evidence of X-gal staining, despite bulky tumor metastases. The second mouse showed moderate positivity for X-gal staining, with some metastases exhibiting no

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staining. Although the outgrowth of b-gal negative tumors
as the cause of death in these long term survivors can not
be concluded, it is a likely possibility.

Example 24

Active Immunotherapy of Established Pulmonary
Metastases with v. B7-2/β-gal and v. MCB7-1/B7-2/β-gal

As stated earlier, optimal T cell activation require
two signals, once delivered by the MHC/TCR interaction and
a second from the B7-1/CD28 or CTLA-4 interaction. It was
evaluated whether a rVV coexpressing B7-2 (v. MCB7-2/β-
gal), or both B7-1 and B7-2 (v. MCB7-1/B7-2/β-gal), with a
TAA would be a more potent vaccine to stimulate TAA
specific CTL response.

Mice were initially inoculated with 5 x 10^5 tumor
cells of CT26.WT or CT26.C25. Mice bearing three day
tumors were then immunized with a single injection of rVV,
with designated groups receiving adjuvant treatment with
IL-2 as outlined above. In addition to the rVV described,
an additional rVV was constructed v. MHA/B7-2/b-gal,
which contained the MHA gene in the Hind III region of the
VV genome, and both B7-2 and the E. coli lac Z gene in the
TK region. This was created to control for the presence
of the B7-1 gene in the v. MCB7-1/B7-2/β-gal construct
(Fig. 20.). No significant tumor regression was seen
following rVV vaccination in mice bearing CT26.WT tumors.

As seen in previous active immunotherapy experiments
with mice bearing three-day CT26.C25 tumors, vaccination
with v. MCB7-1/β-gal resulted in a significant reduction
in the number of pulmonary metastases ($p < 0.0014$), while
v. MCMHA/β-gal mediated only a minimal reduction ($p
< 0.054$), and v. B7-1/NP mediated no reduction ($p < 0.13$).
Vaccination with both v. MCB7-2/β-gal and v. MCB7-1/B7-
2/β-gal resulted in a significant reduction in the number
of pulmonary metastases ($p < 0.0018$ and $p < 0.0018$,
respectively). Similarly, treatment with v. MCMHA/B7-2/β-
gal also mediated significant reduction in the number of